Silencing of Armadillo Repeat-Containing Protein 8 (ARMc8) Inhibits TGF-β-Induced EMT in Bladder Carcinoma UMUC3 Cells

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Armadillo repeat-containing protein 8 (ARMc8) is a key factor in regulating cell migration, proliferation, tissue maintenance, and tumorigenesis. However, its role in bladder cancer remains unknown. Thus, in this study we sought to investigate the effect of ARMc8 on the epithelial-to-mesenchymal transition (EMT) progress in bladder cancer cells induced by transforming growth factor-β1 (TGF-β1). Our results found that ARMc8 was highly expressed in bladder cancer cell lines. ARMc8 silencing inhibited the TGF-β1-induced migration and invasion and suppressed the EMT progress in bladder cancer cells. Furthermore, ARMc8 silencing inhibited the TGF-β1-induced expression of β-catenin, cyclin D1, and c-myc in bladder cancer cells. In conclusion, the present study demonstrates a novel function for ARMc8, which acts as a mediator for TGF-β1-induced cell migration/invasion through modulation of the Wnt/β-catenin signaling pathway in bladder cancer cells. This study suggests that ARMc8 may be a potential therapeutic target for the development of therapies for bladder cancer.

Key words: Armadillo repeat-containing protein 8 (ARMc8); Bladder cancer;
Transforming growth factor-β1 (TGF-β1); Epithelial-to-mesenchymal transition (EMT)

INTRODUCTION

Bladder cancer is the most common malignant tumor affecting the urinary system, and the incidence of bladder cancer has been rising steadily during the past decades (1). Although great progress has been made in diagnostic intensity and treatment, the prognosis for patients with advanced bladder cancer remains dismal (2–4). Thus, the identification of the molecular mechanism during bladder cancer progression may provide patients with novel diagnostic and therapeutic strategies.

Epithelial-to-mesenchymal transition (EMT) is considered to be the key step by which tumor cells gain the higher ability of invasiveness and metastasis. It is characterized by the loss of epithelial characteristics and the acquisition of a motile, invasive, and migratory mesenchymal phenotype (5). Transforming growth factor-β1 (TGF-β1), a multifunctional cytokine, was found to induce EMT in tumor cells (6–8). Therefore, inhibition of TGF-β1-induced EMT may be a therapeutic approach for the treatment of bladder cancer.

Armadillo repeat-containing protein 8 (ARMc8) is a novel armadillo repeat-containing protein that is conserved in eukaryotes. Previous studies have demonstrated that ARMc8 plays an important role in regulating cell migration, proliferation, tissue maintenance, and tumorigenesis (9–11). A study by Jiang et al. showed that ARMc8 promoted the invasiveness and migration capacities of colon cancer cells, while ARMc8 siRNA treatment significantly reduced the invasion and migration in colon cancer cells (12). Zhao et al. reported that knockdown of Armc8 expression significantly inhibited the invasive ability and upregulated E-cadherin expression in HepG2 cells (13). However, its role in bladder cancer remains unknown. Thus, in this study we sought to investigate the effect of ARMc8 on EMT progress in bladder cancer cells induced by TGF-β1.

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MATERIALS AND METHODS

Cell Culture and Treatment

Human bladder cancer cell lines (BIU-87, UMUC3, and 5637) and normal bladder epithelial cell (SV-HUC-1) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 U/ml streptomycin under a humidified incubator that was maintained at 37°C and supplied with 5% CO_2 and 95% air. The bladder cancer cells were then treated with TGF-β1 (10 ng/ml) for different times.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from bladder cancer cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. About 5 μg of total RNA for each sample was reverse transcribed into first-strand cDNA for qRT-PCR analysis. The primer sequences used were as follows: ARMc8, 5′-GTGTAGAGCTGGA GCAATTCGG-3′ (forward) and 5′-AGGCAAGTGTCTC AGCTCCTTCGTCCCCTCGAGGAGTTGTGT-3′ (reverse); and β-actin, 5′-CATGTACGTTGCTATCCAGG C-3′ (forward) and 5′-CTCCTTAATGTCACGCACGAT-3′ (reverse). The steps in the qRT-PCR were performed as follows: 94°C for 2 min for initial denaturation; 94°C for 30 s, 59°C for 15 s, and 72°C for 15 s; 2 s was used for plate reading for 35 cycles; and a melt curve was generated from 65°C to 95°C. For relative quantification, the levels of individual gene mRNA transcripts were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Cells were homogenized and lysed with RIPA lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM EDTA, 10 mM β-glycerophosphate, 2 mM sodium vanadate, and protease inhibitor). Proteins (30 μg per lane) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transfected onto a nitrocellulose membrane (Millipore, Boston, MA, USA). The membrane was blocked with 5% nonfat milk solution for 1 h, followed by incubation with primary monoclonal antibody against ARMc8, E-cadherin, N-cadherin, β-catenin, cyclin D1, and c-myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Following three washes with TBST buffer, the blots were washed and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Protein bands were evaluated by enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, IL, USA).

Transfection of siRNA Against AMRc8

The small interfering RNA against AMRc8 (si-AMRc8) and its negative control siRNA (si-NC) were obtained from Shanghai Sangon Co. Ltd (Shanghai, P.R. China). For in vitro transfection, si-AMRc8 or si-NC was transfected into bladder cancer cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. siRNA knockdown efficiency was verified by Western blot detection of AMRc8 expression.

Cell Migration and Invasion Assays

Cell migration was performed in modified Boyden chambers with 8-mm pore filter inserts in 24-well plates (BD Biosciences, Eugene, OR, USA). Briefly, UMUC3 cells (1×10^5 cells/well) transfected with si-AMRc8 or si-NC were seeded onto the upper chamber containing serum-free culture medium. The lower compartment of the chamber was filled with culture medium containing 10% FBS. The chambers were then cultivated in 5% CO_2 at 37°C for 24 h. All nonmigrant cells were removed from the upper faces of the Transwell membranes with a cotton swab, and cells on the lower filters were fixed with 95% methanol, stained with 0.1% crystal violet, and then counted in five random fields per well under a light microscope (magnification: 100×). The invasion assay was done using the same procedure, except that the membrane was precoated with 100 ml of Matrigel (BD Biosciences, San Jose, CA, USA) at a 1:4 dilution in DMEM to form a genuine reconstituted basement membrane.

Statistical Analysis

All results are reported as means ± SD. Statistical analysis involved using the Student's *t*-test for comparison of two groups or one-way ANOVA for multiple comparisons. A value of $p < 0.05$ was considered to indicate a statistically significant difference.
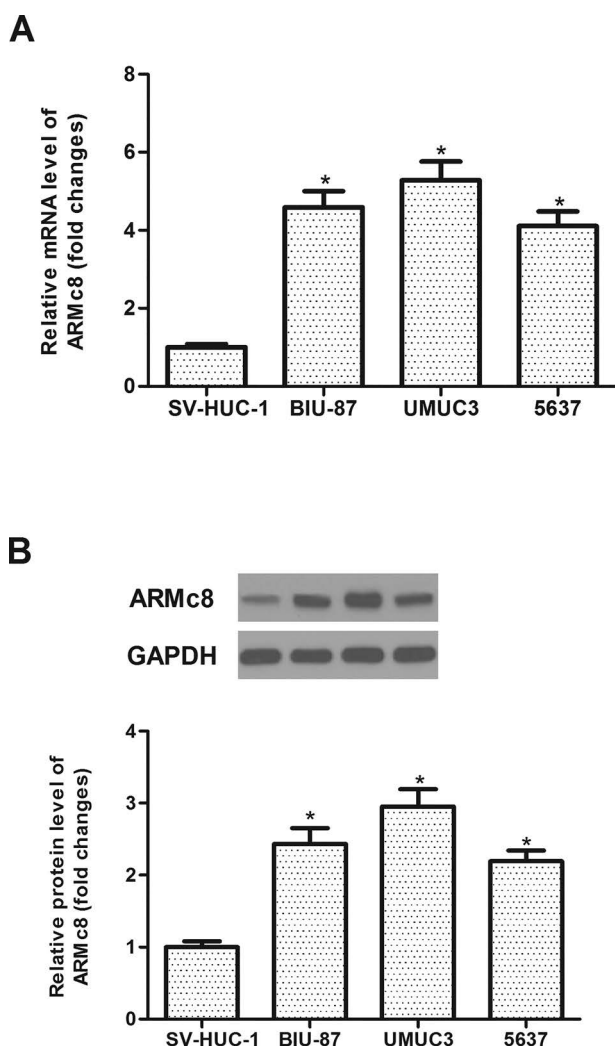
RESULTS

### ARMc8 is Highly Expressed in Bladder Cancer Cell Lines

The expression levels of ARMc8 were detected in human bladder cancer cell lines and human normal bladder epithelial cell line. The results of the qRT-PCR analysis demonstrated that ARMc8 mRNA expression was higher in bladder cancer cell lines than that in normal bladder epithelial cell line (Fig. 1A). Consistent with the results of the qRT-PCR analysis, Western blot analysis showed that ARMc8 protein expression was significantly increased in bladder cancer cell lines, compared with the SV-HUC-1 group (Fig. 1B).

ARMc8 Silencing Inhibits TGF-β1-Induced EMT in Bladder Cancer Cells

To evaluate the effect of ARMc8 in bladder cancer carcinogenesis, we created stable bladder cancer cells with knockdown of ARMc8. The results of the Western blot analysis demonstrated that si-ARMc8 significantly decreased endogenous ARMc8 protein expression in bladder cancer cells, compared with the si-NC group (Fig. 2A). We next evaluated the effect of ARMc8 on EMT progress in bladder cancer cells induced by TGF-β1. As indicated in Figure 2B, TGF-β1 treatment significantly increased the expression of N-cadherin and

Figure 1. ARMc8 is highly expressed in bladder cancer cell lines. (A) The expression of ARMc8 mRNA was evaluated in bladder cancer cell lines and normal bladder epithelial cell line by qRT-PCR analysis. (B) The expression of ARMc8 protein was evaluated in bladder cancer cell lines and normal bladder epithelial cell line by Western blot analysis. *$p<0.05$ versus SV-HUC-1 group.
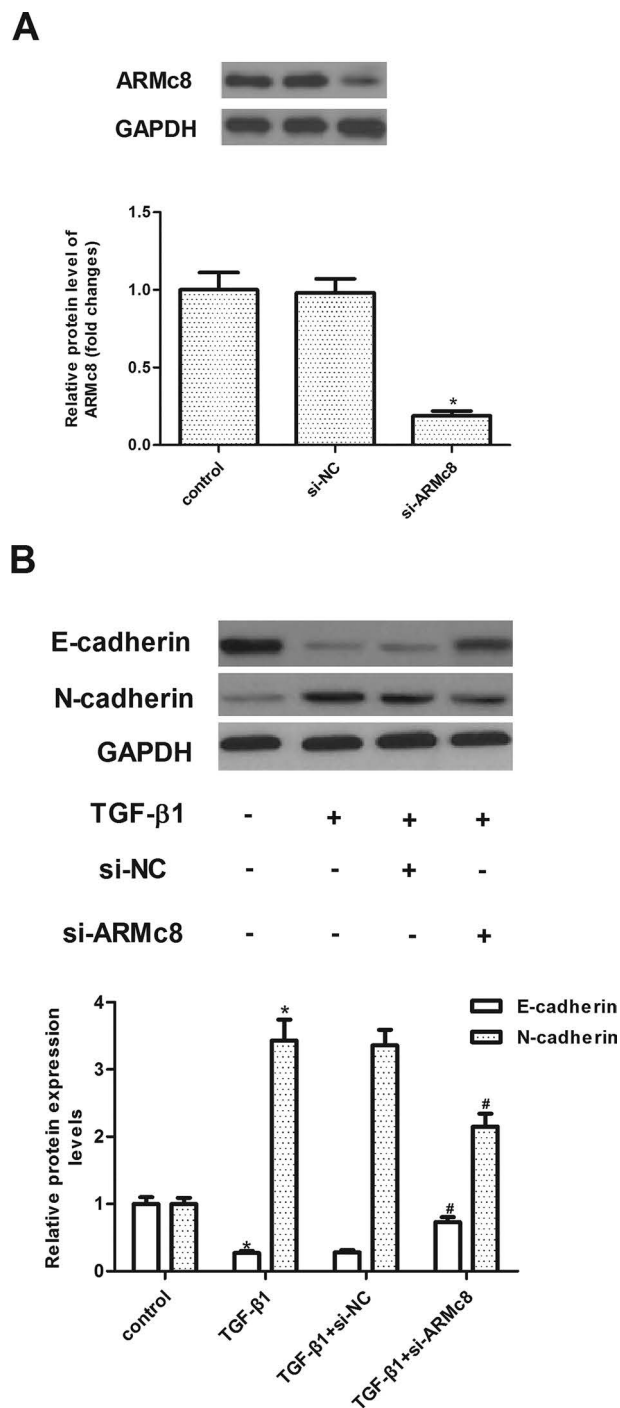
Figure 2. ARMc8 silencing inhibits TGF-β1-induced EMT in bladder cancer cells. (A) The transfection efficiency was confirmed by Western blot analysis in UMUC3 cells after transfection with si-AMRc8. (B) UMUC3 cells transfected with si-AMRc8 or si-NC were treated with TGF-β1 (10 ng/ml) for 24 h. The protein expression levels of E-cadherin and N-cadherin were detected by Western blot analysis. Quantification analysis was performed using the Gel-Pro Analyzer version 4.0 software. *$p<0.05$ versus control group, #$p<0.05$ versus TGF-β1 + si-NC group.

reduced the expression of E-cadherin; however, ARMc8 silencing dramatically inhibited TGF-β1-mediated upregulation of N-cadherin and repression of E-cadherin in UMUC3 cells.

ARMc8 Silencing Inhibits TGF-β1-Induced Migration and Invasion in Bladder Cancer Cells

We performed Transwell assays to measure the effects of ARMc8 on cell migration and invasion in bladder cancer cells induced by TGF-β1. The results revealed that treatment with TGF-β1 greatly promoted UMUC3 cell migration; however, ARMc8 silencing significantly inhibited TGF-β1-induced migration (Fig. 3A). In addition, we observed that ARMc8 silencing also dramatically inhibited UMUC3 cell invasion induced by TGF-β1 (Fig. 3B).

ARMc8 Silencing Inhibits the Activation of Wnt/β-Catenin Signaling Pathway in Bladder Cancer Cells Induced by TGF-β1

To elucidate the mechanism of si-ARMc8-inhibited EMT in bladder cancer cells induced by TGF-β1, we tested the effect of si-ARMc8 on the activation of Wnt/β-catenin signaling pathway signaling. As shown in Figure 4, TGF-β1 treatment significantly increased the expression of β-catenin, cyclin D1, and c-myc in UMUC3 cells. At the same time, ARMc8 silencing obviously inhibited the TGF-β1-induced expression of β-catenin, cyclin D1, and c-myc in UMUC3 cells.

DISCUSSION

In the present study, we found that ARMc8 was highly expressed in bladder cancer cell lines. ARMc8 silencing inhibited TGF-β1-induced migration and invasion, as well as suppressed the EMT progress in bladder cancer cells. Furthermore, ARMc8 silencing inhibited the TGF-β1-induced expression of β-catenin, cyclin D1, and c-myc in bladder cancer cells.

Several studies showed that ARMc8 plays a critical role in the development of cancer. Upregulation of ARMc8 is observed in various tumors. A recent study showed that the expression of ARMc8 was obviously upregulated in ovarian cancer and the bordering ovarian tumor tissues, in comparison with benign ovarian tumors and normal ovarian tissues (14). Elevated expression of ARMc8 was also found in breast carcinoma compared to that in normal breast epithelia (15). In accordance with previous studies, results of the present study demonstrated, for the first time, that ARMc8 is highly expressed in bladder cancer cell lines, suggesting that ARMc8 may be an oncogene in the development and progression of bladder cancer.

EMT is a major phenotype of cancer metastasis and invasion (16). TGF-β1 is the major cytokine that induces EMT during cancer progression and metastasis (17). In line with these results, we observed that TGF-β1 treatment significantly promoted the migration/invasion and EMT progress in bladder cancer cells, and ARMc8 silencing inhibited the TGF-β1-induced migration and invasion, as well as suppressed the EMT progress in bladder cancer cells. These data suggest that ARMc8 silencing inhibited the TGF-β1-induced EMT, consequently affecting bladder cancer cell migration and invasion in vitro.

A

B
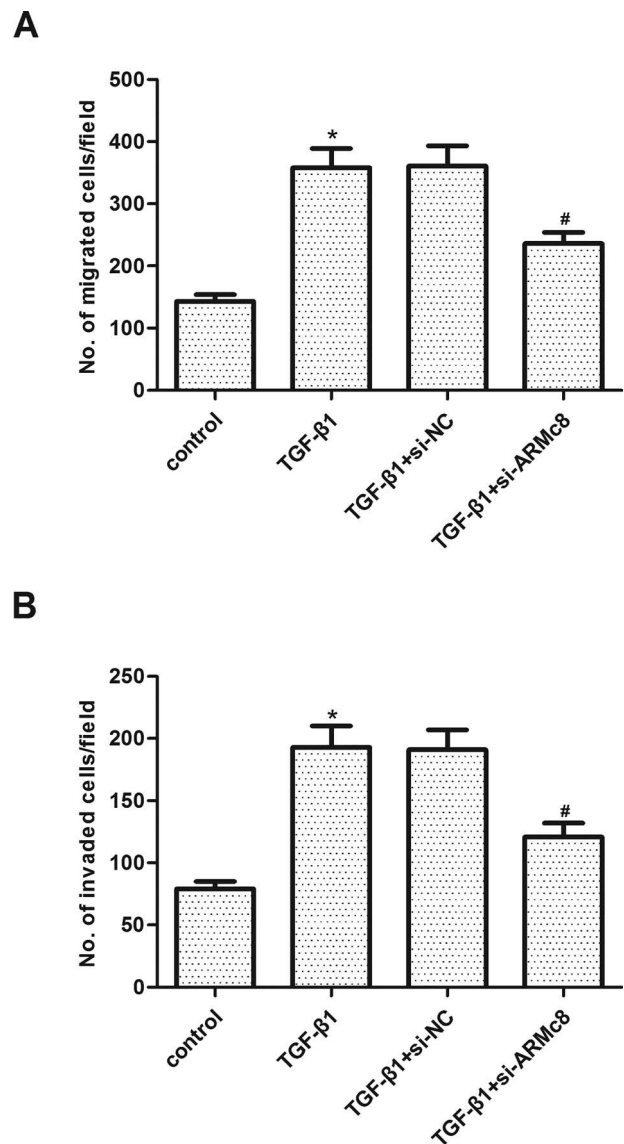
Figure 3. ARMc8 silencing inhibits TGF-β1-induced migration and invasion in bladder cancer cells. UMUC3 cells transfected with si-AMRc8 or si-NC were treated with TGF-β1 (10 ng/ml) for 24 h. (A) Cell migration was detected by Transwell assay. (B) Cell invasion was detected by Transwell assay with Matrigel. *$p<0.05$ versus control group, #$p<0.05$ versus TGF-β1+si-NC group.

A

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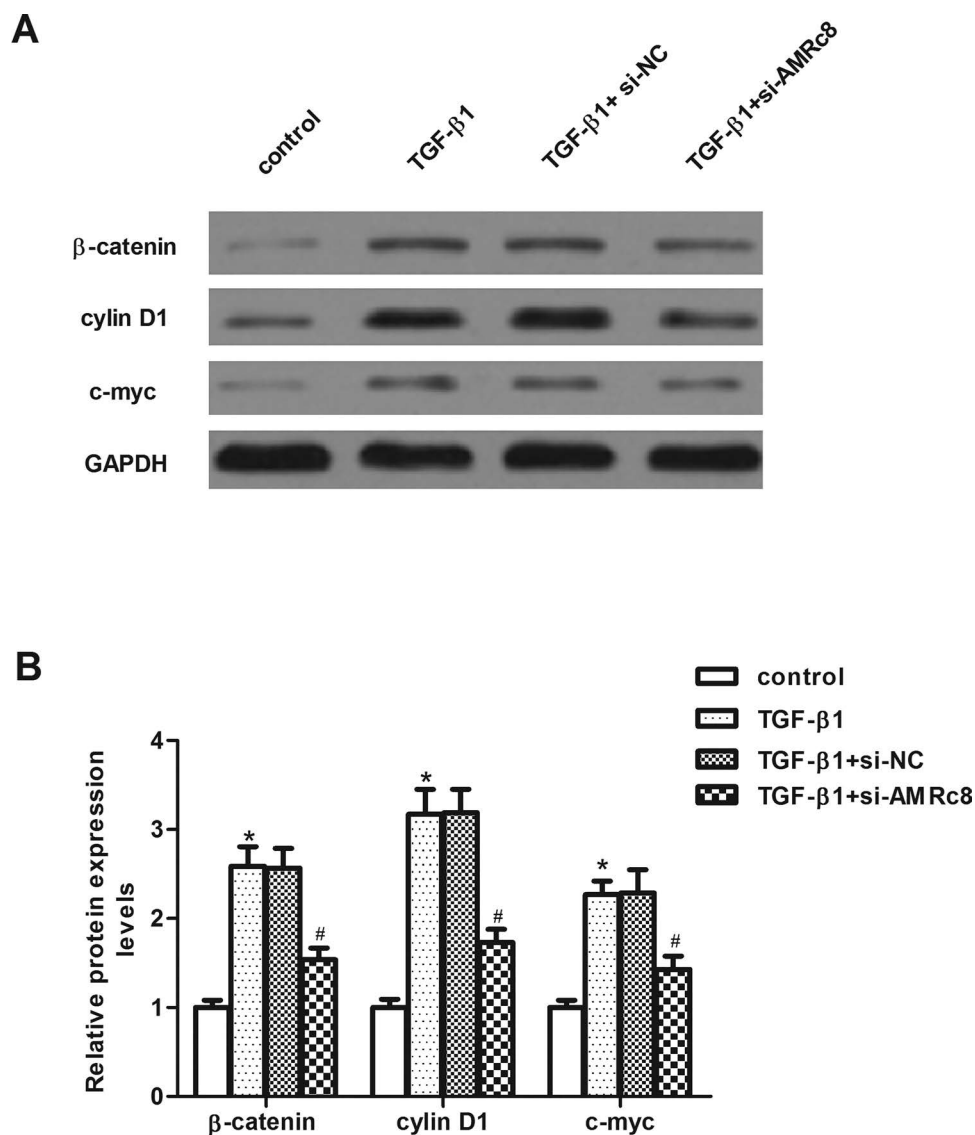
Figure 4. ARMc8 silencing inhibits the activation of Wnt/β-catenin signaling pathway in bladder cancer cells induced by TGF-β1. (A) UMUC3 cells transfected with si-AMRc8 or si-NC were treated with TGF-β1 (10 ng/ml) for 30 min. The protein expression levels of β-catenin, cyclin D1, and c-myc were evaluated by Western blot analysis. (B) Quantification analysis was performed using the Gel-Pro Analyzer version 4.0 software. $*p < 0.05$ versus control group, $\#p < 0.05$ versus TGF-β1 + si-NC group.

The Wnt/β-catenin signaling pathway is well known for its involvement in cancer progression and metastasis. β-Catenin is a critical molecule in Wnt signaling, which is implicated in the pathogenesis of human malignancies (18–20). Several studies showed that nuclear β-catenin was highly expressed in bladder cancer cells (21,22). Activation of Wnt signaling induces the nuclear translocation of β-catenin, which not only initiates a cascade of downstream gene transcription, including cyclin D1, c-myc, and MMP, but also increases the expression of the EMT regulators Snail and vimentin in cancer cells (23). Previous studies have shown that TGF-β1 can induce

β-catenin, and constitutively, active β-catenin can cause cancer cell migration and invasion. Furthermore, pharmacologic inhibition of the Wnt/β-catenin pathway resulted in a blockade of the TGF-β1-induced EMT (24,25). Ren et al. reported that FH535, a reversible Wnt signaling inhibitor, could inhibit the TGF-β1-induced EMT phenotype and tumor metastasis of the A549 cell line both in vitro and in vivo (26). Very interestingly, one study reported that ARMC8α knockdown downregulated canonical Wnt signaling pathway activity and cyclin D1 and MMP-7 expression, and ARMC8α overexpression upregulated canonical Wnt signaling pathway activity and cyclin D1

and MMP-7 expression in human NSCLC cells (27). In this study, we observed that TGF-β1 treatment strongly induced the expression of β-catenin, cyclin D1, and c-myc in bladder cancer cells, while ARMc8 silencing obviously inhibited these effects. These results suggest that ARMc8 silencing inhibits TGF-β1-induced EMT phenotype through inactivation of the Wnt/β-catenin signaling pathway in bladder cancer cells.

In conclusion, the present study demonstrates a novel function for ARMc8, which acts as a mediator for TGF-β1-induced cell migration/invasion through modulation of the Wnt/β-catenin signaling pathway in bladder cancer cells. This study suggests that ARMc8 may be a potential therapeutic target for the development of therapies for bladder cancer.

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